

Simplified Assay for Measuring *Toxoplasma gondii* Immunoglobulin G Avidity

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A *Toxoplasma gondii* immunoglobulin G (IgG) avidity enzyme-linked immunosorbent assay (ELISA) was developed that combines the accuracy of assays based on end point titers and the relative ease of assays based on optical density values. Like published procedures, the new assay's avidity index (AI) was based on differential *T. gondii*-specific IgG reactivity in serum-treated wells washed with urea buffer versus that in wells washed with control buffer; unlike previous assays, however, the IgG reactivity was measured quantitatively using a standard curve. The assay was evaluated using 24 IgG-positive and IgM-positive sera collected within 5 months of the onset of symptoms (recent-infection group) and 25 IgG-positive and IgM-negative sera (past-infection group). All sera in the recent-infection group exhibited AI values of <0.18, whereas all sera in the past-infection group exhibited AI values of >0.27. The AI values of the recent-infection group showed significant correlation with the number of days after the onset of symptoms. A subset of 16 sera (8 recent and 8 past) was tested using a commercially available *T. gondii* IgG avidity ELISA based on end point titration; the results of the two assays showed highly significant correlation ($R^2 = 0.9125$). In addition, we confirmed and extended the findings of other investigators, showing that AI values calculated using optical density values, but not AI values calculated using quantitative IgG values, varied significantly depending on the serum dilution used. This new assay should facilitate the accurate measurement of *T. gondii* IgG avidity in a reference laboratory setting.

Measurement of *Toxoplasma gondii* immunoglobulin G (IgG) avidity (binding strength) is a powerful tool for distinguishing recent from past *T. gondii* infection. Detection of low-avidity IgG is a reliable indicator of infection within the previous 8 months, whereas detection of high-avidity IgG essentially excludes the possibility that infection occurred within the previous 5 months (2, 4, 7). *T. gondii* IgG avidity measurement is particularly valuable for approximating the time of infection in pregnant women found to be positive for *T. gondii* IgG and IgM at the time of their first prenatal care visit (4, 7, 8, 12).

IgG avidity measurement is based on the differential elution of antigen-bound IgG using urea-containing wash buffer; low-avidity IgG dissociates from antigen in the presence of urea, whereas high-avidity IgG remains bound to antigen (2). Results for avidity assays are generally expressed as an avidity index (AI), which reflects the relative amount of *T. gondii*-specific IgG detected following washes with urea buffer versus that found following washes with control buffer. Published methods differ, however, in how these IgG levels are expressed. Using the end point titration method, multiple dilutions of patient sera are tested, and titers are defined in relation to a cutoff optical density (OD) value (2, 4–7). Drawbacks to this method include the requirement for four or more dilutions of a specimen, the frequent need for repeat testing due to insufficient serial dilution of a specimen (i.e., the highest routine dilution gives an OD value higher than the cutoff OD), and

complex calculations. An alternative to end point titration is the OD method, where IgG levels are simply expressed as the OD values obtained following washing with either urea buffer or control buffer (1, 3, 8–11). The OD method, although much easier than the titration method, also has a major drawback: the AI can vary markedly (4), depending on the total amount of *T. gondii*-specific IgG in the specimen. Thus, prior determination of antigen-specific IgG levels is often required before the actual avidity assay can be performed (8–10).

To overcome these drawbacks, we have developed a *T. gondii* IgG avidity assay that incorporates a standard curve, allowing quantitative measurement of *T. gondii* IgG levels using a single dilution of patient serum in most cases. The assay yields AI values comparable to those of the titration method with the relative ease of the OD method.

MATERIALS AND METHODS

Patient sera. The sera ($n = 49$) used for evaluating *T. gondii* IgG avidity were components of the Toxoplasma 1998 Human Serum Panel prepared by the Reference Immunodiagnostic Laboratory, Division of Parasitic Diseases, Centers for Disease Control and Prevention, Atlanta, Ga. These sera were obtained from both men and women, and none of the women were pregnant at the time of blood donation. The sera were divided into two groups on the basis of the time since the onset of toxoplasmosis. The recent-infection group included 24 sera from 11 individuals with primary toxoplasmosis collected within the 5-month period after the onset of symptoms. These patients were infected during an outbreak of toxoplasmosis at a riding stable in 1977 (13); most samples had *T. gondii*-specific IgG levels of >2,000 IU/ml, and all contained *T. gondii*-specific IgM and IgA. The past-infection group included 25 sera collected from individuals who had had toxoplasmosis many months or years earlier (the exact times were unknown), as demonstrated by the presence of *T. gondii*-specific IgG (5 to 1,000 IU/ml) but the absence of *T. gondii*-specific IgM and IgA (13).

Commercially available *T. gondii* IgG avidity assay. A commercially available enzyme-linked immunosorbent assay (ELISA) kit (Labsystems, Helsinki, Fin-

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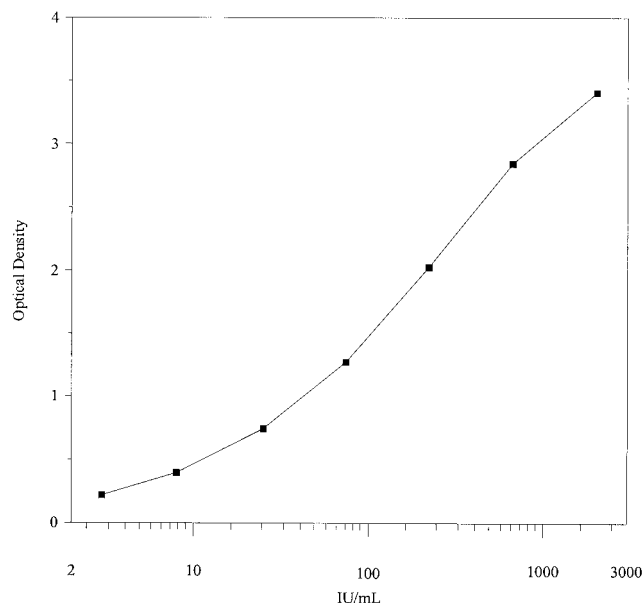


FIG. 1. Representative *T. gondii* IgG standard curve. OD values are plotted on a linear scale versus international units per milliliter on a logarithmic scale.

land) based on the end point titration method was used to measure the avidity of eight recent-infection group sera and eight past-infection group sera. The assay was performed according to the instructions supplied in the kit insert.

Experimental *T. gondii* IgG avidity assay. Microtiter wells (Polysorb; Nunc, Copenhagen, Denmark) were coated with *T. gondii* antigen (Microbix Biosystems, Toronto, Canada) in phosphate-buffered saline (PBS), blocked with PBS containing 0.1% bovine serum albumin (Sigma, St. Louis, Mo.), air dried, and stored at 4°C. Prior to assay setup, the microwell strips were washed with 0.25 ml of control wash buffer (PBS containing 0.1% Tween 20 [Sigma] [PBST]). Patient sera were diluted 1:100 and 1:1,000 in PBST containing 0.1% bovine serum albumin, and each dilution was added to duplicate microtiter wells (0.1 ml per well). Each assay included a seven-point standard curve, prepared by making serial threefold dilutions of the World Health Organization *T. gondii* antibody standard (lot TOXS 60 April 79; Statens Seruminstitut, Copenhagen, Denmark); the curve spanned values of 3 to 2,000 IU/ml. After an hour at room temperature, the well contents were discarded. Control wash buffer was then added to all standard wells, and one of each pair of duplicate wells was treated with a patient serum dilution; control buffer containing 6 M urea (ICN, Aurora, Ohio) was added to the other duplicate well. After 5 min at room temperature, the well contents were discarded and the wash procedure was repeated (including the 5-min soaking step). All wells were washed once more (without soaking) with control wash buffer and then received 0.1 ml of appropriately diluted horseradish peroxidase-conjugated goat anti-human IgG (Fc specific; Jackson Immunore-

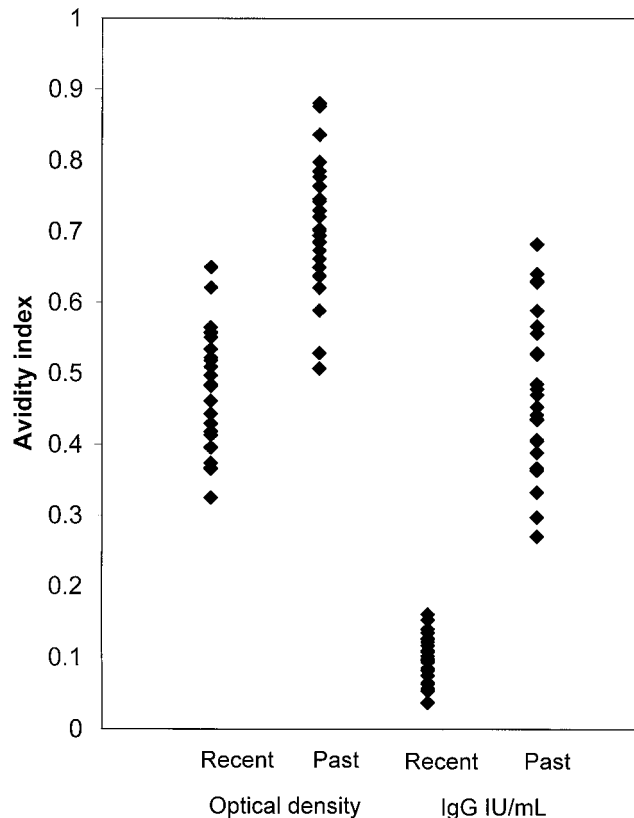


FIG. 2. AI values determined using either OD or international units of IgG per milliliter for sera from the recent-infection group (Recent; $n = 24$) and the past-infection group (Past; $n = 25$).

search, West Grove, Pa.). After 30 min at room temperature, the well contents were discarded and all wells were washed three times with control wash buffer. Substrate reagent (tetramethylbenzidine; Moss Inc., Pasadena, Mo.) was then added to all wells (0.1 ml per well); after 10 min at room temperature, the reaction was stopped by the addition of 0.1 ml of 1 N sulfuric acid (Ricca, Arlington, Tex.). OD values (at 450 nm) were determined using an ELISA plate reader (Bio-Tek Instruments, Winooski, Vt.). The instrument software then plotted the standard curve and used this curve to convert the OD values of serum-treated wells to quantitative IgG values, expressed in international units per milliliter. For a given patient serum dilution, the AI was calculated using the following formula: international units of IgG per milliliter for the well washed with urea buffer/international units of IgG per milliliter for the well washed with control buffer. For comparative purposes, the avidity index was also calculated using OD values rather than international units of IgG per milliliter.

Statistical analyses. Correlations were determined by linear regression analysis, and means were compared using the *t* test. Significance was defined as a *P* value of <0.01 .

RESULTS

A representative standard curve for the experimental *T. gondii* IgG avidity assay is shown in Fig. 1. Representative data generated for a specimen from the recent-infection group and a specimen from the past-infection group are shown in Table 1. As expected, for the control wash, the number of international units of IgG per milliliter for the 1:1,000 dilution was approximately 10-fold lower than the comparable value for the 1:100 dilution, demonstrating good linearity of the standard curve. Numbers of international units of IgG per milliliter obtained for the 1:1,000 dilution were not corrected for this 10-fold

TABLE 1. Representative data for a recent-infection group serum and a past-infection group serum

Sample type and parameter	OD		IgG IU/ml	
	1:100 ^a	1:1,000	1:100	1:1,000
Recent infection				
Urea wash	1.225	0.413	61	8
Control wash	2.767	1.394	963	83
AI	0.443	0.296	0.063	0.096
Past infection				
Urea wash	1.297	0.411	76	9
Control wash	1.866	0.604	129	18
AI	0.695	0.680	0.589	0.500

^a Serum dilution.

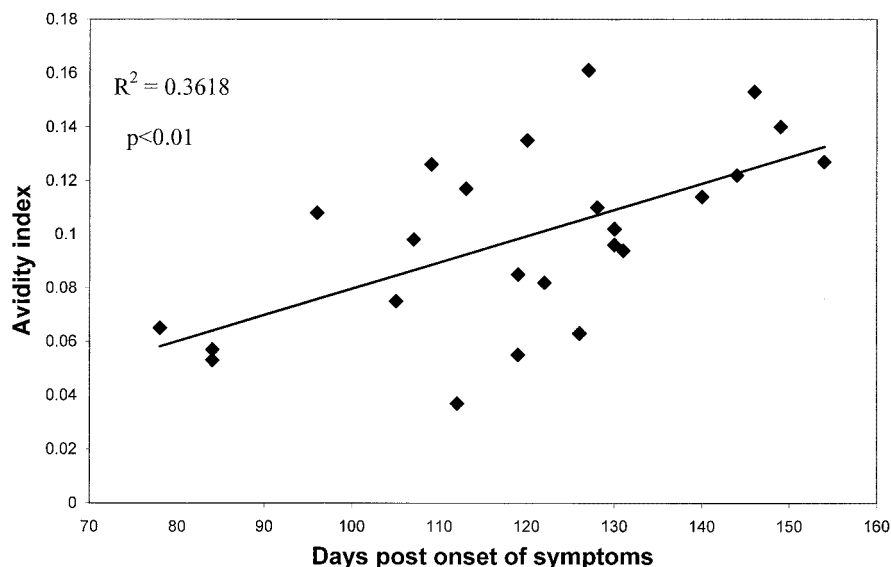


FIG. 3. Relationship between AI based on international units per milliliter and days after the onset of symptoms for the recent-infection group. The R^2 value, determined by linear regression analysis, was statistically significant.

dilution factor, since such correction had no influence on the calculated AI.

An AI based on international units of IgG per milliliter could be calculated using the 1:100 dilution data for all 25 past-infection group sera and 17 of 24 recent-infection group sera. For the seven remaining recent-infection group sera, an AI for the 1:100 dilution could not be calculated because the control wash numbers of international units of IgG per milliliter were greater than the highest standard. However, the 1:1,000 dilution for these seven sera yielded quantitative IgG values that could be used to calculate the AI. Thus, the AI values based on international units of IgG per milliliter pre-

sented in Fig. 2 represent data from the 1:100 dilution for 42 sera (all 25 past-infection group sera plus 17 recent-infection group sera) and the 1:1,000 dilution for 7 sera (all within the recent-infection group). For comparative purposes, the AI values based on ODs for these same serum dilutions are also shown in Fig. 2.

Figure 2 demonstrates the superior performance of AI determination based on international units of IgG per milliliter for distinguishing the recent-infection group from the past-infection group. Although the range of AI values based on OD was obviously lower for the recent-infection group than for the past-infection group, pronounced overlap of ranges was ob-

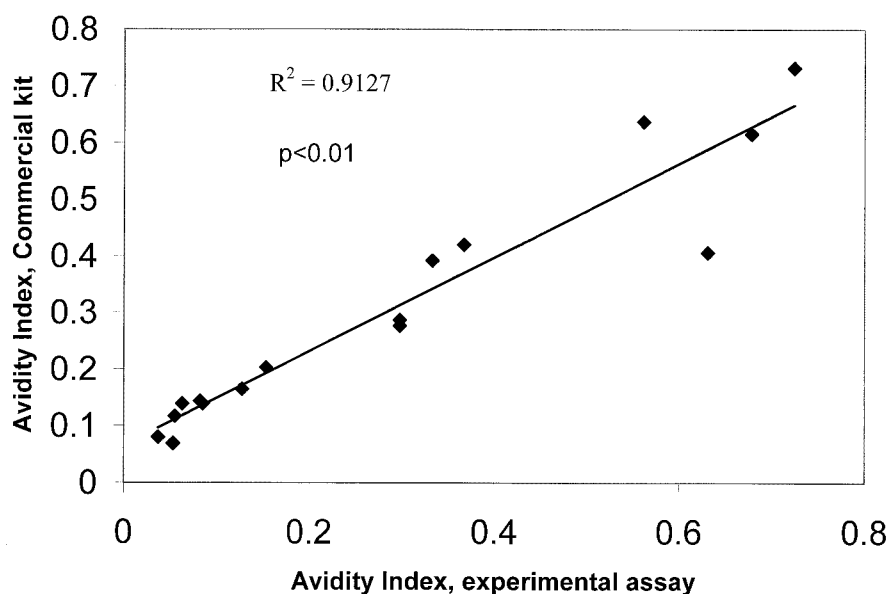


FIG. 4. Relationship between AI values based on international units per milliliter and AI values obtained using a commercially available kit for eight sera from the recent-infection group and eight sera from the past-infection group. The correlation was highly significant.

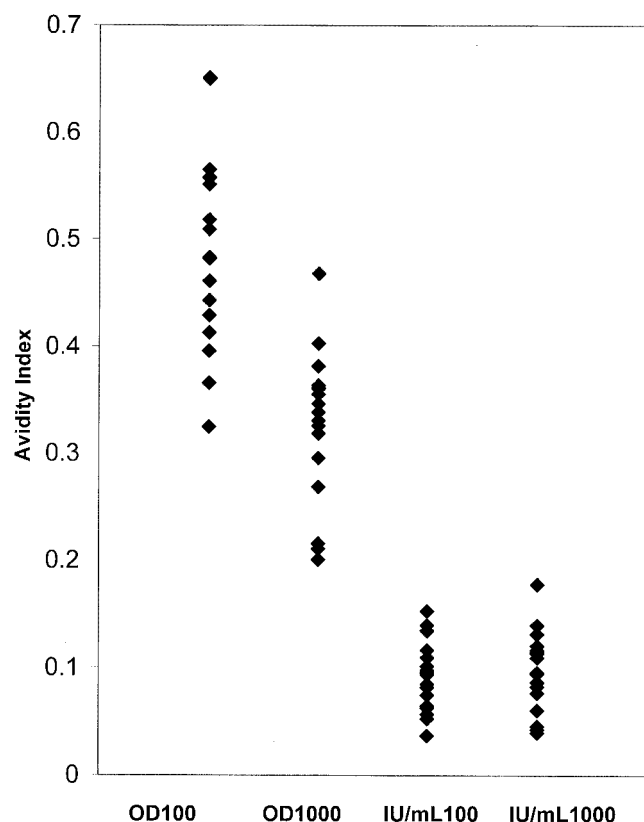


FIG. 5. Influence of specimen dilution on AI values determined using OD values versus quantitative IgG values (international units per milliliter) for 17 specimens from the recent-infection group. The numbers on the abscissa (100 or 1,000) indicate the serum dilution factor.

served. In contrast, no overlap was observed using AI values based on international units of IgG per milliliter; all recent-infection group sera exhibited AI values of <0.18 , whereas all past-infection group sera exhibited values of >0.27 .

The relationship between AI values based on international units per milliliter and days after the onset of symptoms for the recent-infection group is shown in Fig. 3. Increasing time since onset of symptoms was significantly correlated with increasing AI.

A subset of sera (eight recent-infection group and eight past-infection group) was tested using a commercially available *T. gondii* IgG avidity ELISA in which the AI was based on end point titration. In order to compare the results of the commercial assay to those of the experimental assay, the commercial assay results were expressed as the true index (i.e., without multiplication by 100 to enable expression as a percentage). The correlational relationship between AI values (based on international units per milliliter) from the experimental avidity assay and AI values from the commercial assay is shown in Fig. 4; the correlation coefficient was highly significant. Further, the mean AI for the 16 sera as determined in the experimental assay did not differ significantly from the mean AI determined in the commercial assay (0.284 ± 0.060 versus 0.301 ± 0.045 , respectively; $P = 0.370$; paired t test).

As mentioned in the introduction, previous investigators reported that AI values based on the OD for a given specimen

varied markedly depending on the amount of *T. gondii*-specific IgG in the specimen (4). The results shown in Fig. 5 confirm this finding and extend it by showing that such variation does not characterize AI values based on international units per milliliter. These data represent AI values for the 17 recent-infection group sera for which complete OD and international unit per milliliter data were available at both the 1:100 and 1:1,000 serum dilutions. The distribution of AI values based on OD was clearly higher for the 1:100 dilution than for the 1:1,000 dilution; this difference was highly significant when it was evaluated using the paired t test (mean values, 0.490 ± 0.089 versus 0.327 ± 0.071 , respectively; $P < 0.01$). In contrast, the distributions of AI values based on international units per milliliter were very similar at both dilutions, and the mean values did not differ significantly (0.092 ± 0.032 at 1:100 versus 0.097 ± 0.037 at 1:1,000; $P = 0.429$; paired t test). Data are not presented for the past-infection group because most of these sera (18 of 25) had undetectable IgG (defined as <6 IU/ml [4]) for one or both wash treatments at the 1:1,000 dilution.

DISCUSSION

We have developed an ELISA for measuring *T. gondii* IgG avidity that combines the discriminatory power of end point titration assays with the performance ease of OD assays. Rather than titrating each specimen under both urea wash and control wash conditions, a single specimen dilution is tested under both wash conditions, and the IgG content is quantified by comparison to a titrated standard (i.e., the standard curve). This approach avoids the strict graphing and interline measurement steps required for each specimen when the end point titration method is used (2, 7). Further, initial testing of two serum dilutions by the experimental method yielded an AI result for all specimens, obviating the need for repeat testing due to failure to reach an end point titer.

Based on our findings for a panel of well-characterized sera, we consider an AI (based on international units per milliliter) of <0.20 low avidity and an AI of >0.25 high avidity; AI values between 0.20 and 0.25 are considered intermediate avidity. These ranges show excellent agreement with the definitions of low and high avidity for AI values based on end point titration. In seminal publications describing *T. gondii* IgG avidity, Hedman's group considered AI values of $<20\%$ (equivalent to 0.20 in our assay) low avidity and AI values of $>25\%$ (equivalent to 0.25) high avidity (2, 6). Similarly, Jenum et al. considered 20% the transition point for distinguishing high avidity from low avidity (4).

Problems with variations in AI values based on OD in relation to the serum dilution tested have apparently plagued most investigators attempting this approach. As discussed by Jenum et al. (4), several prior reports described assays in which sera were first pretested for *T. gondii* IgG levels and then diluted to a target level (either OD or quantitative units) for performance of the actual OD-based avidity assay. This pretesting requirement thus makes the OD-based avidity assay essentially equivalent to the titration-based assay in terms of technical and mathematical complexity. The new assay described here represents an improvement over both OD-based and titration-based avidity assays, since only two serum dilutions and no pretesting are required.

The recent-infection group sera used in this study were from the 1977 Atlanta stable outbreak (13) and exhibited extremely high levels of *T. gondii*-specific IgG. Such high IgG levels cannot be considered representative of levels found in the typical patient with primary toxoplasmosis (2, 4). In addition, the study did not include sera collected during the very early phase of infection (i.e., days 1 to 78 after the onset of symptoms). Additional studies utilizing sera from more typical toxoplasmosis patients, collected over the full time course of early infection, are needed to demonstrate the utility of this assay in other clinical situations.

The simplified format of this new ELISA for *T. gondii* IgG avidity makes it practical for performance in a reference laboratory setting, where reagent costs and ease of setup are major factors in the selection of new test offerings. Due to its superior clinical utility for estimating the time of *T. gondii* infection (2–12), particularly in expectant mothers, *T. gondii* IgG avidity measurement should be included in infectious-disease testing regimens.

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